

12. Actinomycete taxonomy: generic characterization

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SUMMARY

The taxonomic characterization of novel metabolite- or enzyme-producing actinomycete isolates is an extremely important part of any discovery research program, since it can provide useful insight during later process development. It is also necessary for patent applications that might be submitted. Criteria for the characterization of actinomycete strains to the genus level have become standard over the past few years, and include morphological and chemotaxonomic properties. Changes in recent years have been primarily improvements in analytical procedures. Identification of unknown strains to the species level generally requires a more detailed study of morphological details as well as a varying range of physiological properties, depending upon the genus under investigation. The species concept in many actinomycete genera is still poorly understood, and an evaluation of strains within these genera by classical, numerical, and molecular taxonomic techniques may be necessary to clarify this situation.

INTRODUCTION

Taxonomic characterization of actinomycetes producing novel metabolites is an extremely important step in any screening program. In many instances, however, study of the microorganism is only initiated when the metabolite it produces is identified as being of great interest, i.e., when a description of the producing microbe is needed for the patent application. Rapid identification of isolates, at least to the genus level, can provide valuable information for later stages in development, such as fermentation scale-up. Identification of isolates to the species level will often give the isolation biochemist a clue as to whether or not a metabolite is novel. The use of selective isolation pressures to generate isolates from nature generally will increase

the probability that particular actinomycete genera will be recovered. For many actinomycete genera, the identification to the genus level can be completed in 2–3 weeks.

Actinomycete classification was originally based largely on morphological observations and, although morphology is still an important characteristic for the description of taxa, it is not adequate in itself to differentiate between many genera. The advent of chemotaxonomic criteria for the description of actinomycete genera has provided taxonomists with a set of reliable and reproducible tools for studying the systematics of this group. A general outline for the identification of actinomycete isolates to the genus level is presented in Fig. 1, and will serve as a guide to this discussion.

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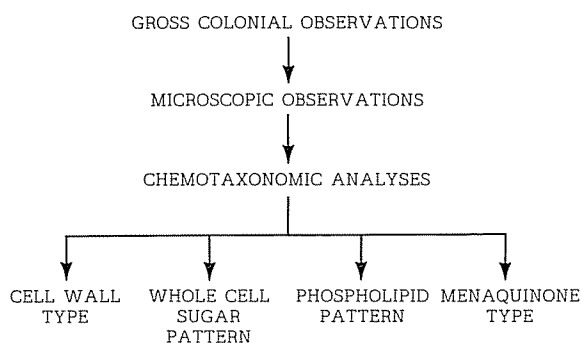


Fig. 1. Flow chart for identification of actinomycetes to the genus level.

DISCUSSION

Morphological observations

Morphology, as previously mentioned, has always been an important characteristic used to identify actinomycete strains, and, in fact, it was the only characteristic used in many early descriptions, particularly of *Streptomyces* species in the first few editions of *Bergey's Manual*. Morphological observations are best made on a variety of standard cultivation media. Several of the media suggested for the International *Streptomyces* Project [28] and by Pridham et al. [26] have proven to be useful in our hands for the characterization of strains accessioned into the ARS Actinomycetales Culture Collection. These media include Czapek's agar, yeast extract-malt extract agar, glycerol asparagine agar, and inorganic salts-starch agar. In order to cultivate the nonstreptomycete 'rare actinomycetes', ATCC Medium No. 172 (NZ-amine glucose starch agar) [2] has been included in the evaluation series. Gross morphological observation of colonial characteristics such as amount and color of vegetative growth, presence and color of aerial mycelium and spores, and presence of diffusible pigments are recorded for each strain studied.

Colonial growth on agar plates can be scanned microscopically under high-dry magnification for the presence of sporulation. The mode of sporulation is important for differentiation between genera, as in the differentiation of *Micromonospora* strains

from *Actinoplanes* strains. Some strains may not sporulate on the usual media used to evaluate gross morphology, and may need to be cultivated on a minimal medium, such as soil extract agar or 2% crude agar in tap water (M.P. Lechevalier, personal communication), in order to induce sporulation. Spores from strains having spores borne on aerial hyphae can be rapidly examined by transmission electron microscopy simply by touching the surface of the sporulated colony with a coated grid and examining the spores directly without further processing. Scanning electron microscopy can provide far more detailed information concerning the sporulation micromorphology of actinomycetes, particularly those whose spore structures are associated with the vegetative mycelium (e.g., *Micromonospora*). Agar blocks containing colonial growth can be cut from plates used for gross morphological observations, fixed in osmium tetroxide vapor overnight, dehydrated, critical point dried and then observed in a scanning electron microscope after mounting and coating. Actinomycete strains can also be grown on glass fiber filters saturated with an agar medium and incubated on the surface of a plate filled with the same medium [1]. The filters can be prepared for scanning electron microscopy in a manner similar to that for agar blocks. Although this method is not as convenient, it does work well for time course studies of sporulation by scanning electron microscopy. Scanning electron microscopy provides information not only on spore surface characteristics, but also on sporophore and spore arrangement, and the presence of a 'sheath' or sporangium surrounding spores.

Chemotaxonomic analysis

Successful actinomycete taxonomists now must be analytical biochemists, as well as microbiologists. Chemotaxonomic criteria are absolutely essential for the differentiation of many actinomycete genera, because some are so similar morphologically, but differ with regard to their diagnostic chemical composition. For example, they differentiate *Nocardopsis* (no characteristic whole cell sugar) from *Saccharothrix* (rhamnose and galactose as characteristic whole cell sugars), or *Amycolata*

(phospholipid pattern PIII, principal menaquinones MK-8 series) from *Amycolatopsis* (phospholipid pattern PII, principal menaquinones MK-9 series).

Cell wall type and whole cell sugar pattern sensu Lechevalier and Lechevalier [18] are the primary chemotaxonomic data that need to be determined, and in some instances may be the only chemotaxonomic data necessary for identification of a strain to the genus level. Air-dried biomass (preferably autoclaved) is required for these determinations by the standard procedure of Becker et al. [3,4]. We have found, however, that the procedure described by Hasegawa [8] works adequately for rapid determination of the diaminopimelic acid isomer present in the cells. In this procedure, several colonies scraped off an agar surface are autoclaved in a sealed tube with 6 N HCl, and the hydrolysate is spotted directly on cellulose thin-layer plates. Thin-layer chromatography with the solvent system of Stanek and Roberts [34] is quite suitable for separation of diaminopimelic acid (DAP) isomers, and is much quicker than downflow paper chromatography. The hydrolysis of cells for whole cell sugar pattern is relatively simple using the procedure of Becker et al. [3,4], but downflow paper chromatography for analysis is a rather slow and cumbersome procedure. The procedure of Stanek and Roberts [34] for thin-layer chromatography of diagnostic sugars on cellulose plates works reasonably well, although it is not able to separate mannose and arabinose. The preparation of trimethylsilyl derivatives of the resulting sugars, followed by gas-chromatographic analysis, has been used by some workers [35,38] and does yield quantitative data, but the derivatization reaction can be a fairly complicated and difficult process. Thus far, no suitable high pressure liquid chromatography (HPLC) method has been found that will permit the resolution of all of the diagnostic sugars. Moreover, refractive index detectors needed to monitor sugars are not sufficiently sensitive. A better procedure, currently under evaluation in our laboratory (unpublished data), is a modification of that of Gauch et al. [7] in which Whatman K5 silica gel plates and an acetonitrile and water solvent system are used.

This is particularly effective when combined with the detection spray of Bounias [5], which is 0.2% *N*-(1-naphthyl)ethylenediamine HCl and 3.0% sulphuric acid in methanol. Following spraying and heating for 5 min at 105°C, the sugars present on the plate can be detected at concentrations as low as 0.15 nmol.

Most actinomycete isolates can be keyed to the appropriate genus after micromorphological observations have been made and cell wall type-whole cell sugar pattern have been determined. The distribution of actinomycete genera based on cell wall chemistry is shown in Table 1. Nonsporulating strains can create identification problems, since they may have cell wall chemistry similar to that of several genera and cannot be positively identified unless they can be induced to sporulate.

Analysis of phospholipid composition of actinomycetes, as described by Lechevalier and Lechevalier [19], is a time-consuming process taking at least 13 days from start to finish. More rapid procedures have been proposed [10,22], but these generally do not detect the glucosamine-containing phospholipids observed using the longer, more detailed procedure. The five phospholipid patterns observed in the Actinomycetales are: PI – no nitrogenous phospholipids present; PII – phosphatidylethanolamine is the only nitrogen-containing phospholipid present; PIII – phosphatidylcholine present; PIV – phosphatidylethanolamine and glucosamine-containing phospholipids present; PV – glucosamine-containing phospholipids are the only nitrogenous phospholipids present [16,17]. The data from these determinations generally serve to confirm the genus assignments made based on sporulation morphology, cell wall type, and whole cell sugar pattern. The phospholipid pattern is important, however, to distinguish between some genera, such as *Amycolata* and *Amycolatopsis*, which have the same general morphology and cell wall type but differing phospholipid patterns. The composition of purified phospholipid components can be further analyzed. Mild deacylation of purified phospholipid fractions releases the fatty acids as methyl esters, which can then be characterized via gas-chromatographic analysis [19]. Sugars present in gly-

Table 1

Distribution of genera of the Actinomycetales by cell wall type, including a description of micromorphology

Type I cell wall

- Streptomyces* – chains of conidia on aerial mycelium.
Streptovercillum – chains or umbels of conidia on verticils formed on aerial mycelium.
Nocardioideis – substrate and aerial mycelium fragment into coccoid elements.
Actinopycnidium – same as *Streptomyces* but pycnidia-like structures formed.
Actinosporangium – same as *Streptomyces* but spores accumulate in drops.
Chainia – same as *Streptomyces* but sclerotia are also formed.
Elytrosporangium – same as *Streptomyces* but merosporangia are also produced on vegetative mycelium.
Intrasporangium – no aerial mycelia; substrate mycelium forms vesicles.
Microellobosporia – merosporangia produced on both aerial and substrate mycelia.
Sporichthya – no substrate mycelium; aerial chains of motile conidia held to surface of substrate by holdfasts.
Kitasatoa – single spores in sporangia on aerial and substrate mycelia; spores motile.

Type II cell wall

- Micromonospora* – no aerial mycelium; single conidia produced.
Actinoplanes – globose-to-lageniform sporangia; motile spores.
Amorphosporangium – same as *Actinoplanes* but irregular sporangia; spores generally non-motile.
Ampullariella – lageniform-to-globose sporangia; motile rod-shaped spores.
Dactylosporangium – claviform sporangia containing one chain of motile spores.
Glycomyces – aerial mycelium with chains of non-motile conidia.

Type III cell wall

No characteristic whole cell sugars

- Actinosynnema* – synnemata with chains of motile conidia.
Geodermatophilus – hyphae divide in all planes, forming packets of motile coccoid conidia.
Nocardiopsis – long chains of conidia on aerial mycelium.
Thermomonospora – single conidia formed on aerial and substrate mycelia.
Thermoactinomyces – single heat-resistant endospores produced on aerial and substrate mycelium.

Madurose as characteristic whole cell sugar

- Actinomadura* – short chains of conidia on aerial mycelium.
Dermatophilus – same as *Geodermatophilus*.
Exellospora – short chains of conidia on aerial and substrate mycelia.
Microbispora – longitudinal pairs of conidia on aerial mycelium.
Microtetraspora – chains of four to six conidia on aerial mycelium.
Planobispora – cylindrical sporangia, each containing two motile spores.
Planomonospora – cylindrical sporangia, each containing one motile spore.
Spirillospora – globose sporangia with rod-shaped motile spores.
Streptosporangium – globose sporangia with nonmotile spores.

Rhamnose and galactose as characteristic whole cell sugars

- Saccharothrix* – long chains of conidia on aerial mycelium.

Type IV cell wall

- Nocardia* – abundant filamentation, often fragmenting into coccoid rods; aerial mycelium and chains of conidia sometimes formed.
Actinopolyspora – long chains of conidia on aerial mycelium; substrate mycelium may fragment.
Amycolata – abundant filamentation, chains of conidia formed on aerial mycelium; substrate mycelium may fragment.
Amycolatopsis – same as *Amycolata*.
Micropolyspora – short chains of conidia formed on aerial and substrate mycelia.
Faenia – same as *Micropolyspora*.
Pseudonocardia – long, cylindrical conidia formed on aerial mycelium, dividing into shorter coccoid elements.
Saccharomonospora – single spores primarily on aerial mycelium.
Saccharopolyspora – similar to *Nocardiopsis*.
Kibdelosporangium – chains of conidia produced on aerial mycelium; sporangia-like structures also produced.

Type X cell wall (cell walls contain glycine and meso and LL isomers of DAP).

- Kitasatosporia* – long chains of conidia produced on aerial mycelium.

colipids can be extracted by acid hydrolysis and analyzed by paper or thin-layer chromatography. These latter two procedures yield more descriptive taxonomic information concerning a strain, but do not generally provide any additional confirmation of the assignment to a given genus.

Menaquinones can be easily isolated from freeze-dried cells by the method of Collins et al. [6], and menaquinones are also present in the chloroform eluate from silica gel columns used in the Lechevalier phospholipid purification procedure [19]. The menaquinones in these extracts are purified by preparative thin-layer chromatography, and analysis is then performed by HPLC. The purified menaquinones are rapidly analyzed by reverse-phase high performance liquid chromatography [13,37]. The identity of peaks observed by HPLC may require confirmation by mass spectroscopy, and the major menaquinones in the purified menaquinone fraction may also be identified by direct mass spectroscopy of this fraction. Menaquinone patterns appear to be somewhat genus-specific and may be used to confirm the assignment of a strain to a given genus.

Analysis for the presence of mycolic acids in actinomycetes having a type IV cell wall sensu Lechevalier and Lechevalier can be important in distinguishing members of the genus *Nocardia* from the genera *Amycolata* and *Amycolatopsis*. The standard procedure for analyzing cells for mycolate content involves extraction of biomass with methylene chloride, followed by the preparation of methyl esters of the mycolates and analysis by gas chromatography [19]. A number of short methods are proposed in the literature for the analysis of mycolates, including the gravimetric procedure of Kanetsuna and Bartoli [11], the LCN-A test of Mordarska et al. [23], and the procedure of Hecht and Causey [9]. The LCN-A test of Mordarska et al. has proven useful in our laboratory for analysis of an occasional nocardioform strain requiring evaluation.

Identification to the species level

The characteristics used to delineate species are largely dependent upon the genus being studied. Morphological observations performed to identify

an unknown strain to the genus level also provide data useful in assigning strains to a species within that genus. Speciation of strains within most genera of the Actinomycetales has been based upon morphological and physiological characteristics. Morphological characteristics that are used for species identification include the color of substrate mycelium, the presence and color of aerial mycelium, color of spores, shape and ornamentation of fruiting bodies and spores, and production and color of soluble pigments. A wide range of physiological characteristics have been evaluated, including carbohydrate utilization profile, nitrogen source utilization profile, degradation or hydrolysis of numerous substrates, and sensitivity to various inhibitors. The delineation of species within many actinomycete genera is subject to change as additional tests for the species differentiation are developed, and can lead to differences of opinions among taxonomists.

A good example of the differences that can exist in species definition is found with the genus *Streptomyces*. Classically, species of this genus are described by the characteristics of spore color and ornamentation, sporophore morphology, aerial and substrate mycelium color, production of melanin and diffusible pigments, and utilization of the carbohydrates D-glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, D-galactose, raffinose, D-mannitol, *i*-inositol, salicin, and sucrose. These characteristics were used in the International *Streptomyces* Project studies [29–32], and the collaborators were able to describe over 450 species of *Streptomyces* in the course of the ISP program. This study did not attempt to compare or differentiate between species, although several workers did attempt to develop identification keys from this database [12,24,36]. A compilation of the number of taxa from this study that are recognized as valid *Streptomyces* species in the approved list of bacterial names [33] and of those validly published thereafter results in a total of 346 species, described primarily in terms of the classical criteria. By contrast, Pridham [25] proposed a 'lumping' classification scheme, based solely on spore ornamentation and sporophore morphology, which reduced

the genus *Streptomyces* to eight species. This greatly simplifies the identification process but is hardly satisfactory for the definitive descriptions required for publication or patent purposes. The study of Williams et al. [39] undertook the herculean task of examining a total of 475 strains, including the type strains of 394 *Streptomyces* species, for 139 unit characters, which included both morphological and physiological characteristics. The results were examined by numerical taxonomic methods, and the *Streptomyces* type strains were found to fall into 19 major and 40 minor clusters, including 18 single-member clusters at the 77.5% simple matching similarity level. It is their interpretation that these single-member clusters and the remaining minor clusters containing from two to five strains probably represent species. They proposed that the remaining major clusters, ranging in size from six to 71 strains, be considered as species-groups until further information is available. The cluster groups produced by this study are of great concern to classical *Streptomyces* taxonomists, because only one major and nine minor clusters were homogeneous with regard to spore color, and spore surface ornamentation appears to be of limited diagnostic value. The 77 cluster groups can be distinguished from each other, and strains can be assigned to clusters based on only 41 tests [40], but this classification system will probably not be well accepted by industrial *Streptomyces* taxonomists since, in its present form, it is not well suited to describe strains for patent purposes. The results of this study could be further validated through evaluation of the DNA pairing between strains within cluster groups, the most powerful tool. The genetic validity of these phenetic 'species' could be determined, and a combination of both phenotypic and genetic data could provide the most useful classification scheme.

CONCLUSIONS

There have been no major changes in the criteria used for the identification of actinomycete isolates in the past several years, although there have been improvements in some analytical technology. Fur-

ther improvements can be expected in the future. The application of the criteria described by Lechevalier and Lechevalier in 1980 [19] to the identification of actinomycete isolates has resulted in the creation of the new genera *Amycolata* [20], *Amycolatopsis* [20], *Glycomyces* [15], *Saccharothrix* [14] and *Kibdelosporangium* [27]. Species that were misplaced in inappropriate genera have also been reclassified to the appropriate genera through the use of these chemotaxonomic standards. The species concept in many genera of the Actinomycetales is still poorly defined, but application of chemotaxonomic, numerical taxonomic, and molecular taxonomic techniques will result in clarification of the systematics within these genera.

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